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2-ETHOXYCARBONYL-2-METHYL-3,4-DIHYDRO-*2H*-PYRROLE-1-OXIDE: EVALUATION OF THE SPIN TRAPPING PROPERTIES.

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Abstract: The 2-ethoxycarbonyl-2-methyl-3,4-dihydro-*2H*-pyrrole-1-oxide (EMPO), an easily prepared pyrroline-N-oxide has been tested as free radical scavenger. Spin adducts of superoxide, hydroxyl radical and other free radicals were characterized in phosphate buffer at pH 7.0 and 5.6. At pH 7 in phosphate buffer, the EMPO/$O_2^{-\bullet}$ spin adduct was estimated to be about 5 times more persistent than its DMPO analogue. Furthermore, its decay does not produce the EMPO/ $HO^{\bullet}$ adduct.

Keywords: spin trapping, superoxide, hydroxyl radical, nitrones.

INTRODUCTION

The involvement of reactive oxygen species in physiological and pathological processes is still under considerable investigation[1]. A large number of spin trapping studies have been devoted to understand the role played by oxygen-centered radicals in these processes[2]. The most widely used spin trapping agent was for a long time the DMPO (2,2-dimethyl-3,4-dihydro-2*H*-pyrrole-1-oxide) **1**[3]. However, the use of DMPO underwent some limitations, such as its sensitivity to nucleophilic attack of water and the relatively low persistence of its superoxide spin adduct, DMPO/$O_2^{-\bullet}$, which decomposes to give the hydroxyl adduct, DMPO/$HO^{\bullet}$[3d, 4]. We previously reported the synthesis and spin trapping properties of a new nitrone, the 2-diethoxyphosphoryl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (DEPMPO) **2**[5]. DEPMPO can be easily prepared by a two step-synthesis[6] and it was shown to trap efficiently oxygen-centered radicals. In phosphate buffer at pH7.0, the persistence of the DEPMPO/$O_2^{-\bullet}$ superoxide adduct is approximately 15 times higher than that of DMPO/$O_2^{-\bullet}$ and the consecutive formation of DEPMPO/$HO^{\bullet}$ was not observed. Furthermore, in phosphate buffer (0.1 M) DEPMPO does not undergo nucleophilic addition of water in presence of Fe^{3+} (1 mM). Then, in order to clarify the role played by the phosphorus moiety in the stabilization of the superoxide spin adduct, we prepared the 2-(diethoxyphosphorylmethyl)-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide **3** in which the

phosphorus group is separated from the nitrone moiety by a methylene spacer[7]. The ESR experiments driven with **3** showed that the overall characteristics of this nitrone in the spin trapping of oxygen-centered radicals are closer to that of DMPO rather than to that of DEPMPO, thus indicating the importance of the strong electron-withdrawing effect of the phosphorylated group[8] in the stabilization of the DEPMPO/$O_2^{-\bullet}$spin adduct. This observation prompted us to prepare the 2,2-bis(diethoxyphosphoryl)-3,4-dihydro-2*H*-pyrrole-1-oxide **4** [9] (scheme 1). The superoxide spin adduct **4**/$O_2^{-\bullet}$ was well characterized in H_2O_2 30 % and could be observed in phosphate buffer at pH 6.0, but not at pH 7.0. Kinetic decay analysis could be performed in pyridine, using a lumiflavin-light-DTPA system and, in these conditions, the persistence of **4**/$O_2^{-\bullet}$ was found to be similar to that of DMPO/$O_2^{-\bullet}$. The short half-life of **4**/$O_2^{-\bullet}$ is presumably a consequence of the high steric constraint resulting from the presence of the *gem*-bis(diethoxyphosphoryl) groups and from their competition for the preferential pseudo-axial position [9]. In the present paper, we report the results of our work on the synthesis of the 2-ethoxycarbonyl-2-methyl-3,4-dihydro-*2H*-pyrrole-1-oxide **5** (EMPO) (scheme 1) and on the characteristics of this nitrone as free radical trap.

Insert scheme 1

EXPERIMENTAL PROCEDURES

Synthesis

General. NMR spectra were recorded on a Bruker AC 200 spectrometer (^{1}H, 200 MHz; ^{13}C, 50.32 MHz). Mass spectra and HRMS were performed at the University of Rennes (France). Preparative TLC was performed on Merck "Silicagel 60, 230-400 mesh".

Ethyl(4-formyl-2-methyl-2-nitro)butanoate (6). A mixture of ethyl 2-nitropropanoate (0.3 g; 2.3 mmol), acrolein (0.2 g; 3.8 mmol) and triethylamine (0.02 g; 0.2 mmol) in acetonitrile (1 g; 24.4 mmol) was stirred at 10 °C for 1 h. Diluted hydrochloric acid (0.5 ml in 15 ml of water) was then added. The mixture was extracted with methylene chloride and dried over sodium sulfate to yield, after filtration and reconcentration, 98 % (4.61 g) of **6** as a colorless liquid. $\delta_H(CDCl_3)$ 1.30 (3H, *t*, J = 7.2, C*H*$_3$CH$_2$O); 1.79 (3H, *s*, C*H*$_3$C*CH$_2$); 2.4 - 2.7 (4H, *m*, CC*H*$_2$C*H*$_2$CHO); 4.28 (2H, *q*, J = 7.2, CH$_3$C*H*$_2$O); 9.76 (1H, *s*, CHO); $\delta_C(CDCl_3)$ 13.5 (*C*H$_3$CH$_2$O); 21.4 (*C*H$_3$C*O); 28.4 (C*C*H$_2$CH$_2$CHO); 38.1

(C*C*H$_2$*C*H$_2$CHO); 62.8(CH$_3$*C*H$_2$-O); 91.5 (CH$_3$*C**CH$_2$); 166.7 (C*CO$_2$CH$_2$); 199.1 (CHO). Rf : 0.73 (methylene chloride / ethanol 19/1).

2-Ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (5). 0.8 ml of an aqueous solution of ammonium chloride (1.4 g in 6ml of water) was added to a solution of **6** (0.5 g; 2.5 mmol) in a 6/4 v/v H_2O-MeOH mixture. Zinc dust (8.4 g; 12.9 mmol) were slowly added (0.5 h) and the mixture was then left under stirring at room temperature for 4.5 h. The sample was filtered and the residue washed with methanol (5 x 3 ml). The liquid layer was reconcentrated to 1 ml, then saturated with borax, extracted twice with 6 ml of methylene chloride, dried over sodium sulfate, and then filtered and reconcentrated. Preparative TLC of the crude compound (methylene chloride- ethanol 18.5/1.5 v/v, extraction with methanol) afforded 0.2 g (52 %) of pure nitrone **5**. δ_H(CDCl$_3$) 1.30 (3H, *t*, J = 7.1, C*H*$_3$CH$_2$O); 1.70 (3H, *s*, C*H*$_3$C*CH$_2$); 2.1 - 2.3 and 2.5 - 2.8 (4H, 2*m*, CC*H*$_2$C*H*$_2$-C=); 4.24 and 4.26 (2H, 2*q*, J = 7.1, CH$_3$C*H*$_2$O); 7.0 (1H, *t*, J = 2.6, CH$_2$C*H*=N); δ_C(CDCl$_3$) 13.3 (*C*H$_3$CH$_2$O); 20.2 (*C*H$_3$C*O); 25.3 (C*C*H$_2$CH$_2$C=); 31.7 (CCH$_2$*C*H$_2$-C=); 61.5(CH$_3$-*C*H$_2$-O); 78.3 (CH$_3$*C**CH$_2$); 134.7 (CH$_2$-CH=N); 169.2 (C**C*O$_2$CH$_2$). Rf : 0.39 (methylene chloride / ethanol 19/1). HRMS (Calc. for $C_8H_{13}NO_3$: 171.0895. Found: 171.0896. *m/z* 98 ((M - $CO_2Et)^+$, 100 %), 82 (64.37); 57 (21.49); 55 (38.28); 41(36.06); 29 (Et, 50.01); 28 (96.14); 18 (40.91).

Spin trapping experiments

General. Xanthine oxidase (XO) and bovine erythrocyte superoxide dismutase were purchased from Boerhinger Mannheim Biochemica Co. Gluthathione peroxidase, diethylenetriamine pentaacetic acid (DTPA) and other chemicals were obtained from Sigma Chemical Co. Phosphate buffers were stirred for 4 h in presence of a chelating iminodiacetic acid resin (4g per 100 ml) in order to remove traces of metal impurities. ESR spectra were recorded on a computer controlled Varian E-3 ESR spectrometer and on a Bruker ESP 300 spectrometer, equipped with a NMR gaussmeter for field calibration. The UV photolysis was produced by a 1000 W xenon-mercury Oriel lamp. The ESR spectra were simulated with the ESR software developed by D. Dulling from Laboratory of Molecular Biophysics NIEASCN[10] and the ESR simulation program of A. Rockenbauer from the Institute of Chemistry of Budapest[11].

HO$^\bullet$ trapping- Fenton reaction system. HO$^\bullet$ was generated from a standard Fenton system: $FeSO_4$ (2 mM) was added to a solution of 0.1 M nitrone, 2 mM H_2O_2 and 1 mM

DTPA in 0.1 M phosphate buffer. The ESR spectrum of the spin adduct was recorded 40 s after addition of $FeSO_4$.

$H_3C^{\bullet}$, $O_2C^{-\bullet}$ or $O_3S^{-\bullet}$ trapping. A Fenton reaction system was used in presence of 100 mM DMSO, 50 mM sodium formate or sodium sulfite in 0.1 M phosphate buffer, pH 7.0. Recording of the ESR spectra started 40 s after addition of ferrous sulfate.

Superoxide trapping : a) Hypoxanthine-xanthine oxidase system (HX-XO). The ESR solution contained 100 mM nitrone, 1mM DTPA, 0.4 mM hypoxanthine and 0.4 U mL^{-1} xanthine oxidase in 0.1 M phosphate buffer. Oxygen was bubbled into the reaction mixture for 30 s and the ESR spectrum was recorded 40 s after addition of xanthine oxidase. **b) H_2O_2 photolysis.** The superoxide adduct was generated by UV photolysis in the ESR cavity of a solution of 50 mM nitrone in 30 % H_2O_2.

$GS^{\bullet}$ trapping. $GS^{\bullet}$ was produced by UV photolysis of a 50 mM solution of gluthathione disulfide, in presence of 50 mM nitrone.

Kinetics of decay of superoxide spin adducts. Superoxide was generated with the HX-XO system. The nitrone (50 mM) was incubated in a 0.1 M phosphate buffer, pH 7.0, containing hypoxanthine (0.4 mM), xanthine oxidase (0.08 U mL^{-1}), and DTPA (1 mM). After incubation, the production of spin adduct was stopped by addition of superoxide dismutase (2500 U mL^{-1}). The decay of the spin adduct was followed by monitoring the decrease of an appropriate ESR line. Computer simulations were performed as previously reported[5c] using the home-made DAPHNIS simulation program[12].

RESULTS AND DISCUSSION

Synthesis

The synthesis of nitrone **5** was first reported by Bonnett[13]. In the present work, we used a modified procedure as presented in scheme 2.

Insert scheme 2

ESR studies

No artefactual ESR signals were observed at physiological pH for solutions of **5** in phosphate buffer. The ESR characteristics of spin adducts of **5** are reported in Table 1.

Spin trapping of the hydroxyl radical. The hydroxyl radical was produced by a Fenton system (H_2O_2-$FeSO_4$) in 0.1 M phosphate buffer at pH 7.0 or by UV photolysis of a 1 % aqueous solution of H_2O_2. In presence of **5**, the ESR signal (Figure 1a, b) can be attributed to two diastereomeric adducts (52/48 %). It is worth noticing that this stereochemical result dramatically differs from the one observed for the phosphorylated analogue DEPMPO/$HO^{\bullet}$ adduct, for which only one diastereomer was detected[5b]. This difference certainly traduces the smaller size of the 2-ethoxycarbonyl group of **5**, compared to the diethoxyphosphoryl group of DEPMPO. When the Fenton system was used in presence of DMSO, sodium formate or sodium sulfite, the **5**/$H_3C^{\bullet}$ (two diastereomers) (Figure 1c, d) and **5**/$O_2C^{-\bullet}$ or **5**/$O_3S^{-\bullet}$spin adducts were observed instead of **5**/$HO^{\bullet}$. For the two latter adducts, satisfactory simulations could be achieved assuming the presence of only one diastereomer. In phosphate buffer (0.1 M, pH 7.0), nitrone **5** did not undergo nucleophilic addition of water in presence of Fe^{3+} ($FeNH_4(SO_4)_2$, 21 mM)

Spin trapping of superoxide. The superoxide was generated from the hypoxanthine-xanthine oxidase (HX-XO) system in phosphate buffer at pH 5.6 and 7.0. The ESR spectra of **5**/$O_2^{-\bullet}$ (Figure 2a, b) is very distinguishable from that of the hydroxyl adduct and, as for nitrone **3**, only one diastereomer could be detected at the two pH values. The ESR spectra exhibit rather asymmetrical external doublets, and the best simulations were achieved taking into account a two-site conformational exchange. Such asymmetry has been reported even for DMPO/$O_2^{-\bullet}$[14] but is greatly enhanced in the case of DEPMPO/$O_2^{-\bullet}$[5b]. This can be explained by the presence of the large phosphorus coupling constant, as it was shown in the case of *gem*-β-bisphosphorylated nitroxides[15]. The assignment of this signal to the superoxide adduct was supported by its inhibition when adding superoxide dismutase (85 U mL^{-1}) to the generating system and also by its reduction to **5**/$HO^{\bullet}$ in presence of gluthathione-gluthathione peroxidase (10 U mL^{-1}) (GSH-GSH Px). Furthermore, the decay of **5**/$O_2^{-\bullet}$ is not accompanied by the formation of the hydroxyl adduct, and this behavior is similar to that of

DEPMPO/$O_2^{-\bullet}$, but not of DMPO/$O_2^{-\bullet}$ or **3**/$O_2^{-\bullet}$. **5**/$O_2^{-\bullet}$ was also produced by UV photolysis of H_2O_2 (30 %), but with a poorest quality of the resulting ESR signal.

Spin trapping of GS$^\bullet$. The gluthathionyl radical GS$^\bullet$ was produced from UV photolysis of gluthathione disulfide in phosphate buffer at pH 5.6 and pH 7.0. The ESR spectra (Figure 2c, d) exhibit broad lines and are very similar at the two pH values. Satisfactory simulation of this signal was obtained assuming the existence of two diastereomers.

Insert Table 1

Insert Figure 1

Kinetics of decay of the superoxide adduct. Kinetic decay experiments were performed in 0.1 M phosphate buffer, at pH 7.0. The spin trap was incubated (2.5 min) with hypoxanthine-xanthine oxidase, in the presence of DTPA. Then, the formation of the superoxide spin adduct was stopped by adding superoxide dismutase (2500 U mL^{-1}). The decay of **5**/$O_2^{-\bullet}$ was followed by monitoring the decrease of the intensity of the first low field ESR line. The kinetic was simulated as a pseudo-first-order process. Calculated half-life for **5**/$O_2^{-\bullet}$ in these conditions was (4.8 ± 1.1) min. Under the same conditions (except incubation time: 4 min), a half-life of (14.7 ± 1.0) min was found for DEPMPO/$O_2^{-\bullet}$, which was previously shown to be 15 times more persistent than DMPO/$O_2^{-\bullet}$ in phosphate buffer, pH 7.

CONCLUSION

EMPO (**5**) is an easy prepared nitrone which is stable at physiological pH in phosphate buffer. Its superoxide spin adduct EMPO/$O_2^{-\bullet}$ is significantly more persistent than the DMPO analogue and its decay does not generate the EMPO/HO$^\bullet$ spin adduct. EMPO could be more a more interesting tool than DMPO to investigate the fate of different free radicals in biological processes.

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Table 1 ESR characteristics of spin adducts of nitrone **5**.

Spin adduct	Source	%	A_N/G	$A_{H\beta}$/G	$A_{H\gamma}$/G	Exchng time/ns
5/HO$^{\bullet}$	H_2O_2-$FeSO_4$[a*]	53	14.0	15.1	0.9	-
		47	14.0	12.7	-	
5/HO$^{\bullet}$	H_2O_2(1% in water), $h\nu$ [*]	57	14.2	14.2	1.2	-
		43	14.1	12.7	-	
5/$H_3C^{\bullet}$	H_2O_2-$FeSO_4$-DMSO[a *]	83	15.4	22.6	-	-
		17	15.4	21.7	-	
5/$O_2C^{-\bullet}$	H_2O_2-$FeSO_4$-HCO_2Na[a]	-	14.8	17.2	-	-
5/$O_3S^{-\bullet}$	H_2O_2-$FeSO_4$-$HNaSO_3$[a]	-	13.7	15.1	0.4	-
5/$O_2^{-\bullet}$	HX-XO[a **]	48	13.3	8.7	-	90
		52	13.3	12.8	-	
5/$O_2^{-\bullet}$	HX-XO[b **]	48	13.3	8.8	-	96
		52	13.3	12.7		
5/$O_2^{-\bullet}$	H_2O_2(30%), $h\nu$ [**]	53	13.3	7.6	-	28
		47	13.4	16.6		
5/GS$^{\bullet}$	GSSG, $h\nu$ [a*]	65	14.4	14.8	0.4; 0.7	-
		35	14.4	17.7	0.4; 0.7	

[a] 0.1M phosphate buffer, pH7.0. [b] 0.1M phosphate buffer, pH 5.6. * two diastereomers. ** two-site conformational exchange.

Legends

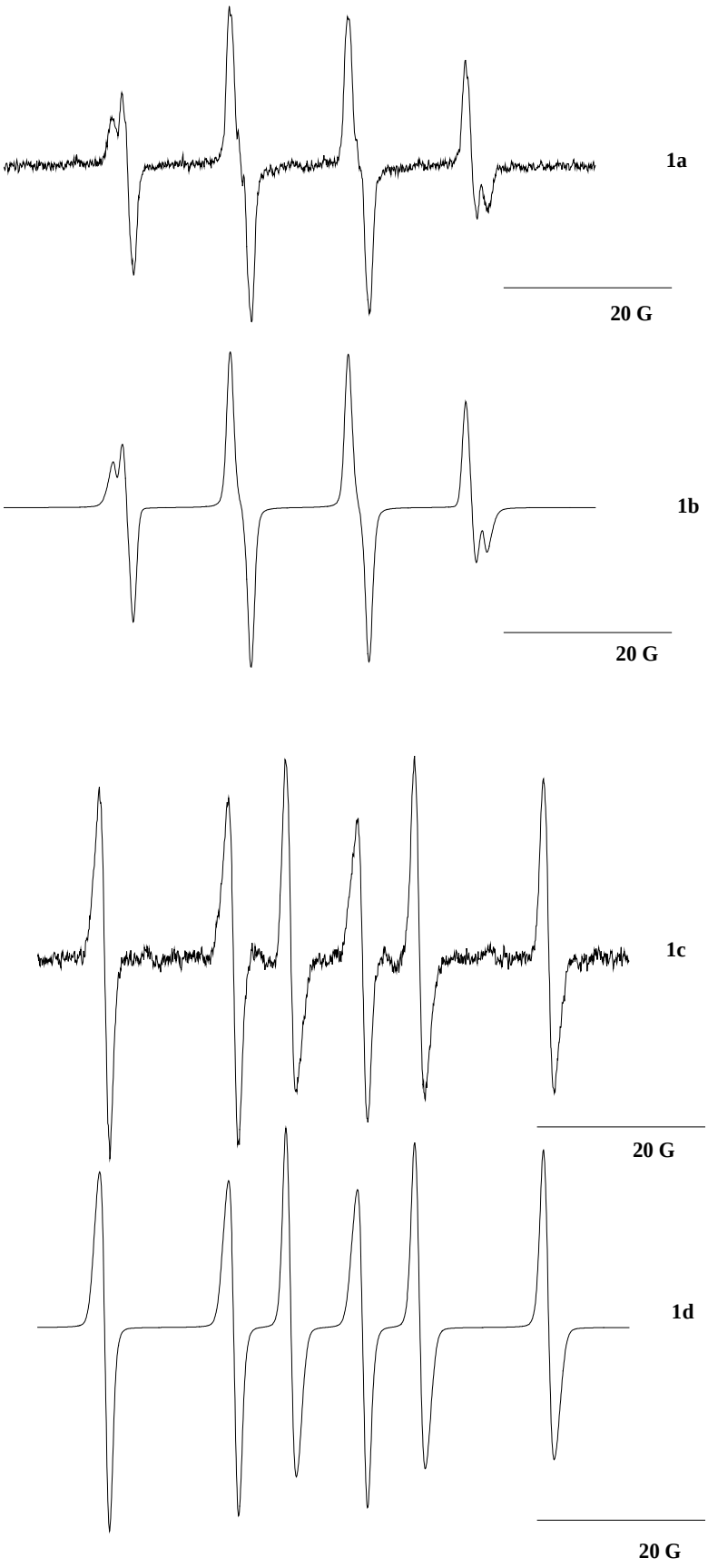
Scheme 1

Scheme 2

Table 1 ESR characteristics of spin adducts of nitrone **5**.

Figure 1: ESR spectra of spin adducts **5**/$HO^{\bullet}$(1a: exp.; 1b: calc.) and **5**/H_3C (1c: exp.; 1d: calc.) in phosphate buffer, pH 7.0.

Figure 2: ESR spectra of spin adducts **5**/$O_2^{-\bullet}$(2a: exp.; 2b: calc.) and **5**/$GS^{\bullet}$ (2c: exp.; 2d: calc.) in phosphate buffer, pH 7.0.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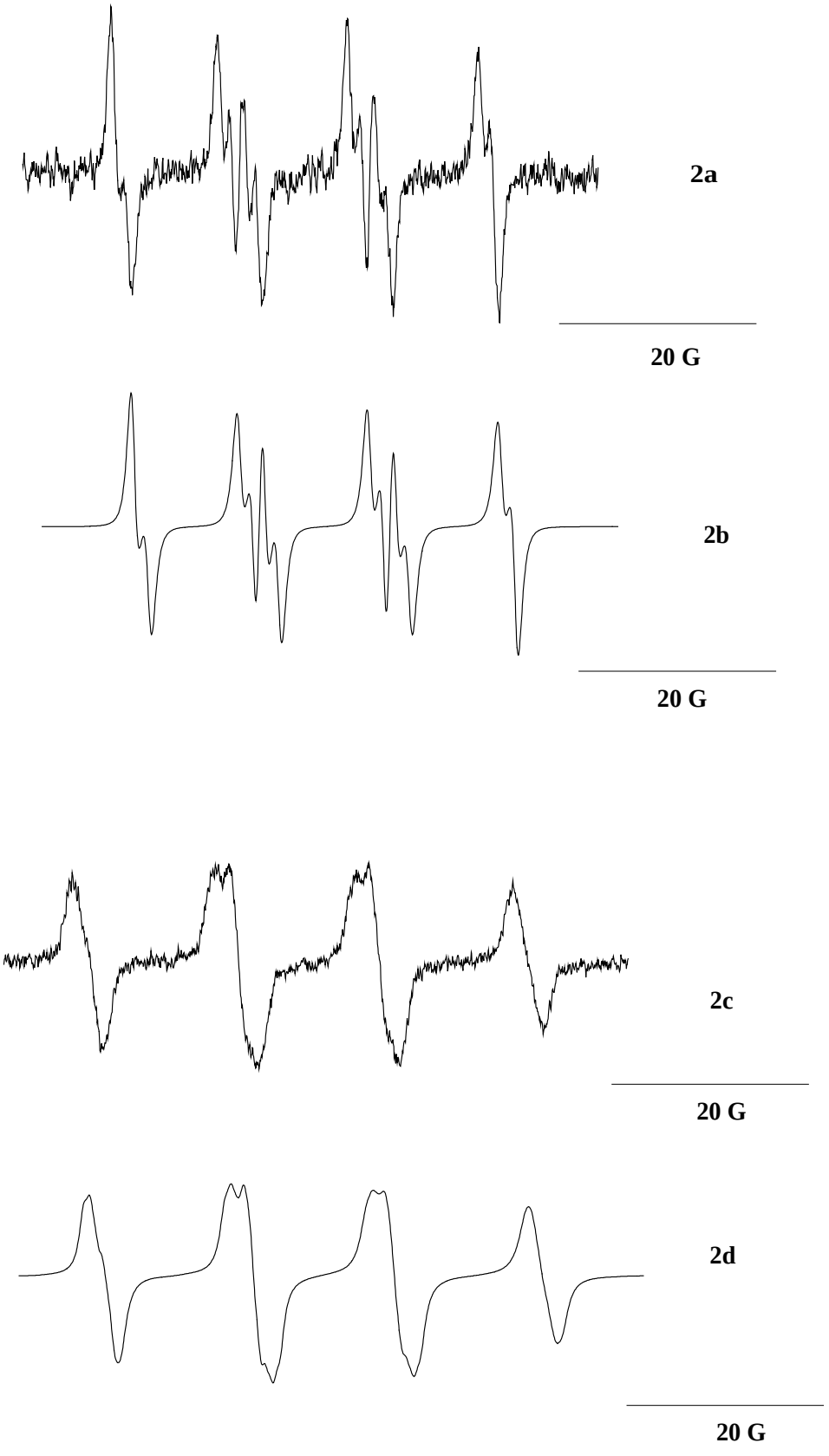
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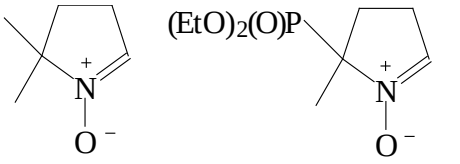

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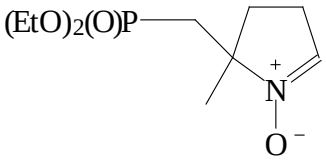

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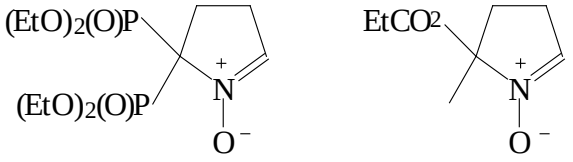
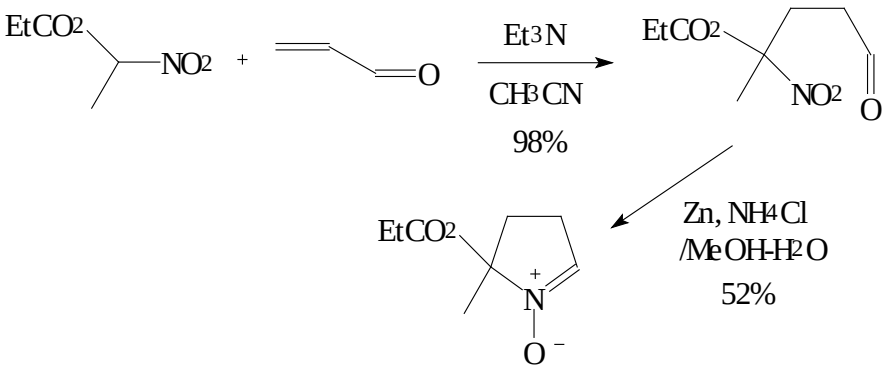


4 **5**

scheme 1

+



scheme 2